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Floral epidermis and phylogeny in the Nymphaeaceae

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Disentangling historical signal and pollinator selection on the micromorphology of flowers: an example from the floral epidermis of the Nymphaeaceae

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Abstract

- The family Nymphaeaceae includes most of the diversity among the ANA-grade angiosperms. Among the species of this family, floral structures and pollination strategies are quite varied. The genus *Victoria*, as well as subgenera *Lotos* and *Hydrocallis* in *Nymphaea*, presents night-blooming, scented flowers pollinated by scarab beetles. Such similar pollination strategies have led to macromorphological similarities among the flowers of these species, which could be interpreted as homologies or convergences based on different phylogenetic hypotheses about the relationships of these groups.

- We employed SEM of floral epidermis for seven species of the Nymphaeaceae with contrasting pollination biology to identify the main characters of the floral organs and the potential homologous nature of the structures involved in pollinator attraction. Moreover, we used TEM to observe ultrastructure of papillate-conical epidermis in the stamen of *Victoria cruziana*. We then tested the phylogenetic or ecological distribution of these traits using both consensus network approaches and ancestral state reconstruction on fixed phylogenies.

- Our results show that the night-blooming flowers present different specializations in their epidermis, with *Victoria cruziana* presenting the most elaborate floral anatomy. We also identify for the first time the presence of conical-papillate cells in the order Nymphaeales. The epidermal characters tend to reflect phylogenetic relationships more than convergence due to pollinator selection.

- These results point to an independent and parallel evolution of scarab pollination in Nymphaeaceae, and show the promise of floral anatomy as a phylogenetic marker. Moreover, they indicate a degree of sophistication in the anatomical basis of cantharophilous flowers in the Nymphaeales that diverges from the most simplistic views of floral evolution in the angiosperms.

Key words:

Cuticle, conical-papillate cells, flower morphology, hydropotes,* Nymphaeaceae, *secretory epidermis

Flowers are the most stunning and diverse feature of the angiosperms. The evolution of floral morphology is linked with the evolution of pollination strategies, with different pollinations vectors being traditionally associated with convergent floral "syndromes" (Fenster et al. 2004). One important aspect of floral morphology which is associated with pollinator attractions is the micromorphology of the floral organs, particularly the perianth (Whitney et al. 2011). Indeed, the morphology and function of petal epidermis has been shown to have an important role in the interaction between pollinators and flowers. For example, the importance of conical-papillate cells on the abaxial side of the petals in the interaction with pollinators has been first shown in the *mixta* mutants of *Antirrhinum majus* L. (Whitney et al. 2009). Such traits have been shown to be associated with pollination mode at large taxonomical scales (Costa et al. 2017) as well as among closely related species (Ojeda et al. 2012), and pollinator shifts have been shown to correlate with shifts in petal micromorphology in an entire biogeographical region (Ojeda et al. 2016). This and other micromorphological traits of the petals have been linked with pollinator attraction or interaction by means of physical effects, i.e. interaction with light, temperature or grip of insects (Moyroud & Glover 2017; Whitney et al. 2011), as well as by their involvement in the production of scent compounds (Marinho et al. 2014). Even if most of these studies have been focused on the petals, the micromorphology of other parts of the flower (or the inflorescence in case of large pseudanthia and/or traps) can also affect the interaction with

different pollinators (Bröderbauer et al. 2013). In the so-called "early diverging angiosperms" (Endress 2011), where the differentiation between organ series is less clear than in the eudicots or most monocots, the inner tepals as well as the stamens and staminodes are also involved in the attraction and the interaction with pollinators (Endress 2010; Thien et al. 2009). However, the micromorphological basis of such interaction in the stamens and staminodes have not been investigated so far.

The family Nymphaeaceae (waterlilies) includes most of the diversity of the early divergent angiosperm order Nymphaeales. Members of this family are widely distributed around the globe, and present striking levels of morphological diversity (Borsch et al. 2008). The family is divided in five genera: the early-diverging *Nuphar* Sibth. & Sm., the small genus *Barclaya* Wall., the closely related annuals *Victoria* Lindley and *Euryale* Salisb., and the large genus *Nymphaea* L.. This last genus includes most of the species of the Nymphaeaceae, and it is subdivided into a boreal clade (subgenus *Nymphaea*) and four tropical clades. Two of these subgenera include species with night-blooming flowers and are vicariantly distributed one in the palaeotropics (subsgenus *Lotos*) and another in the neotropics (subgenus *Hydrocallis*). The other two subgenera include species with day-blooming flowers, with one pantropical subgenus (subgenus *Brachyceras*) and the other restricted to Australia (subgenus *Anecphya*, which includes the species previously segregated in the genus *Ondinea*).

The pollination ecology of the Nymphaeaceae presents interesting patterns. Two vicariant subgenera of *Nymphaea* (subg. *Hydrocallis* and *Lotos*) and the genus *Victoria* present night-blooming flowers which produce a significant amount of scent compounds (Maia et al. 2014) and are pollinated by beetles of the tribe Cyclocephalini (Coleoptera, Scarabeidae) (Ervik & Knudsen 2003). The flowers in these groups tend to be large Chamber blossoms (*sensu* Bernhardt 2000), that scarab beetles use as brooding sites. In *Victoria,* the chamber is formed by extensions of the carpel tips (Fig 1) and it's closed by inner staminodes (paracarpels). In

subgenera *Hydrocallis* and *Lotos,* the chamber is mostly formed by erect stamens (Ervik & Knudsen 2003).

On the other hand, the other subgenera of *Nymphaea* as well as members of the genus *Nuphar* are mostly pollinated by diurnal pollinators. In subgenera *Brachyceras* and *Anecphya* of *Nymphaea,* the stamens curve on top of the stigmatic cup, and act as a slippery surface that prevents insects from escaping the liquid-filled stigmatic cup (Thien et al. 2009). Our understanding of the evolution of pollination strategies in the Nymphaeaceae has been improved by the new views on the phylogeny of this group which have emerged from the analysis of molecular data. When taxon sampling became extensive enough to allow the monophyly of the genus *Nymphaea* to be tested, it became clear that support for such a hypothesis was lacking (Borsch et al. 2007, Borsch et al. 2008). Analysis of fast-evolving chloroplast markers retrieved a clade including *Euryale* plus *Victoria* as sister to *Nymphaea* subg. *Hydrocallis* and *Lotos* (Lohne et al. 2007), while different taxon and gene sampling supported the monophyly of the tropical subgenera of *Nymphaea*, and the sister relationship between such clade and the *Victoria* and *Euryale* clade (Borsch et al. 2008). Such scenarios would imply a potential homology of the pollination syndromes in *Victoria* and the night-blooming *Nymphaea*, and a scenario of pollination niche conservatism in these lineages (Ervik & Knudsen 2003).If an extremely densely sampled analysis of the trnT-trnF spacer did support a monophyly of Nymphaea (Borsch et al. 2011), full chloroplast sequences support a polyphyletic *Nymphaea,* with *Victoria* related to the tropical Nymphaeas (Grünstäudl et al. 2017).

The previous analyses investigating the evolution of morphological characters in the family have retrieved elevated levels of homoplasy, especially in floral characters. Indeed, if characters from leaf architecture seem to present a strong phylogenetic signal (Taylor & Gee 2014), many floral characters (i.e. floral size and number of organs, floral colour) are strongly

linked with pollination mode, and generate a signal which strongly conflicts with the molecular signal (Borsch et al. 2008). This hinders the recognition of genuine synapomorphies for the main clades supported by molecular data, and suggests a strong role for selection from pollinators and a high degree of evolvability in the floral morphology of the family.

Regarding the evolution of the pollination ecology in the family, morphological data could offer an independent line of evidence to disentangle its evolutionary history. The presence of similar structural specializations in *Victoria* and the night blooming *Nymphaea* could strengthen the hypothesis of homology between the pollination strategies of these species. On the other hand, a substantially different micromorphologial basis for the organs involved in pollinator attraction could point towards a parallel evolution of pollination strategies in the family.

Our study aims to characterize the epidermis of the floral organs in the Nymphaeaceae in a sample of members of the family from different clades and with contrasting pollination ecologies. Using these data, we will test two competing hypotheses (Fig. 1F):

1) The structure of the floral epidermis is phylogenetically conserved, with similar anatomical traits in close species with contrasting pollination strategies. Under this hypothesis we expect that the small, cleistogamous flowers of *Euryale* should present very similar anatomical characters to the large, cantarophilous flowers of *Victoria*, and that the anatomical characters of the floral epidermis should present a strong phylogenetic signal.

2) The structure of the floral epidermis is determined by pollination strategy, with similar anatomical specialization in species with similar pollinators. Under this hypothesis, we expect the large, scented flowers of *Victoria* and the night-blooming

Nymphaeas to present similar anatomical specializations, potentially correlated with scent production.

Material and methods

Species studied and Plant material

Sepals, petals and stamens were collected from mature flowers of *Nymphaea alba* L. (subgen. *Nymphaea*) (XX-0-NAP-2730), *Nymphaea caerulea* Savigny (subg. *Brachyceras*) (XX-0-NAP-278) and *Victoria cruziana* A. D. Orb. (XX-0-NAP-2732), stamens were collected from *Nuphar lutea* (L.) Sm. (XX-0-NAP-2731) cultivated at the Botanical Garden of Naples, Italy. Anthers of *Nymphaea gigantea* Hook. (subg. *Anecphya*) (XX-0-BR-19662012) were obtained from the collection in the Jardin Botanique National de Belgique, in Meise, Belgium. Further samples of sepals, petals and stamens were collected from mature flowers of *Nymphaea lotus* L. (accession number 19965454 0U, IPEN XX0Z-19965454), *Euryale ferox* Salisb. (accession number 20150989 P0G,IPEN XX-0-STGAL-2146), and *Victoria cruziana* (accession number 20150988, IPEN XX-0-STGAL-21500) cultivated at the Botanical Gardens of Zurich, Switzerland. Vouchers are deposited at the Herbarium Neapolitanum (NAP) and the Herbarium of the University of Zurich (Z) (Table S1).

Scanning electron microscopy (SEM)

Sepals, petals, stamens collected from *Nymphaea alba*, *N. caerulea*, and stamens of *N.gigantea*, *Nuphar lutea* (L.) Sm, and respectively sepals, petals, staminodes, stamens, paracarpels and carpel tips collected from *Victoria cruziana* were fixed in ethanol 50 % at 4-7°C for at least a week, dehydrated in a graded ethanol series, critical point-dried in liquid CO_2 and coated with approximately 25 nm of gold. The fixation with ethanol reduces costs and toxicity of the reagents used producing, at the same time, excellent samples for SEM

observation. Sepals, petals and stamens collected from *Nympheaea lotus* and sepals, petals, staminodes, stamens, and carpel tips collected from *Euryale ferox* were placed in ethanol 50 %, dehydrated in a graded ethanol series, critical point-dried in liquid CO_2 and coated with approximately 4 nm of platinum. Specimens were examined using a FEI-Quanta 200 scanning electron microscope at an accelerating voltage of 25 kV (*N.caerulea, N.alba, N.gigantea, V.cruziana*) or with a Zeiss Supra 50VP at an accelerating voltage of 15 kV.

Transmission electron microscopy (TEM)

Dissected anthers of *V. cruziana* were fixed in 3 % (v/v) glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 overnight at 4°C, post-fixed in 1% (w/v) OsO_4 overnight at 4°C, and dehydrated through an ethanol series. They were then embedded in Spurr's resin and sections cut at 70 nm using a Reichert-Jung Supernova ultramicrotome. Sections were collected on 200 mesh uncoated copper grids, stained for 12 min in uranyl acetate, post-stained for 8 min in lead citrate and examined using a FEI/ Philips EM 2088 transmission electron microscope at an accelerating voltage of 80 kV at the Laboratory of Measures in Electron and Confocal Microscopy of the Centre of Advanced Metrological Services of the University of Naples Federico II, Naples, Italy.

Signal dissection and morphological congruence

We coded three characters from the epidermal data we produced (Appendix 1). We then generated a series of matrices through integration of our characters with characters taken from Borsch et al. (2008),Taylor et al. (2015) and Coiro & Barone Lumaga (2013); the combinations are explained in Table S2. We predict that if the anatomy of the flower reflects pollination ecology, the amount of tree-like signal in the combined datasets should decrease, and the support for ecological groupings (i.e. *Victoria* plus the night-blooming Nymphaeas)

should increase. We included the pollen data, which present a high degree of homoplasy (Taylor et al. 2015) as a "positive control" of this first prediction. On the other hand, an increase in tree-likeness and a stronger support for clades supported by molecular analyses would indicate a congruence between anatomical traits and historical signal.

To test the congruence of our characters with other morphological characters in the Nymphaeales and the presence of conflicting signals, we employed a consensus-network approach using Maximum Parsimony bootstrap replicates and Bayesian Inferences posterior trees (Coiro et al. 2017) as well as a split-network approach (Denk & Grimm 2009).

The Maximum Parsimony (MP) analyses were conducted using PAUP* 4.0 beta 10 (Swofford 2003). Heuristic search was performed for 100 replicates, using random addition of new taxa and Tree-Bisection-Reconnection branch swapping. Bootstrap analysis was performed using the "as-is" option for addition of the taxa and 1000 replicates. Consensus networks of the bootstrap replicates were generated using SplitsTree v 4 (Huson & Bryant 2006) showing all splits with > 0.10 posterior probability.

The Bayesian Inference (BI) analysis were conducted using MrBayes v3.2.6 (Ronquist et al. 2012). Matrices were analyzed using both equal rates across characters and gamma-distributed rate variation. Two runs of four chains (including three cold chains) were conducted for 1'000'000 generations, sampling every 1000. After discarding the first 25% as burnin, we generated both 50% consensus trees and a consensus network showing all splits with > 0.10 posterior probability. SplitsTree v 4 was used to calculate Delta scores (Holland et al. 2002) on versions of the matrices with all ambiguous characters transformed in uncertainties.

Ancestral character state reconstruction

We used our observations as well as data compiled from the literature (Endress 2008; Warner et al. 2008; Zini et al. 2017) to code epidermal characters at the generic level for *Victoria, Euryale* and *Nuphar,* at the familial level for Cabombaceae, and the subgeneric level for *Nymphaea.* These data were used in an ancestral state reconstruction analysis with a Parsimony criterion using the software Mesquite (Maddison & Maddison 2017). We tested the homoplasy and distribution of our characters on three different topologies representing the conflicting signal in the molecular data of the Nymphaeales: the first topology represents a tree with a monophyletic *Nymphaea,* with subgenus *Nymphaea* sister to *Lotos* plus *Hydrocallis* and *Brachyceras* plus *Anecphya* (Borsch et al. 2011); the second topology represents a tree in which *Victoria* plus *Euryale* are sister to the tropical *Nymphaea* clade (Borsch et al. 2008); the third topology represents a tree in which *Victoria* plus *Euryale* are sister to the night-blooming *Nymphaeas* (Lohne et al. 2007).

Terminology

For the species studied characters of epidermis, presence or absence of hydropotes and stomata were recorded both of abaxial and adaxial surfaces of sepal, petals and staminodes; given the laminar nature of the stamens in Nymphaeaceae and the presence in some species of an apical extension of the connective (tip), information from connective as well as tip surfaces was recorded for these organs (Table 1). Epidermis terminology according to Carpenter (2005) and Koch, Bhushan & Barthlott (2008); hydropotes terminology according to Carpenter (2006).

Data availability

All matrices and trees are available on FigShare at https://figshare.com/s/a74f2a7c92fd366805e7.

Results

Nuphar lutea *(L.) Sm.*

The stamensurface in *Nuphar lutea* shows tetragonal-elongated, slightly convex pavement cells with lightly wavy anticlinal walls. Stomata and hydropotes are absent (Fig. 2A).

Euryale ferox *Salisb.*

The abaxial surface of the sepals in *Euryale* presentstetragonal tabular pavement cells. It is densely covered in hydropotes and bears numerous stomata(Fig. 2B).Some areas appear to be tomentose, and fully covered in uniseriate multicellular trichomes.The adaxial surface differs from the abaxial for the density of hydropotes, which are quite sparse, and for the apparent absence of stomata(Fig. 2C).

The petal surface presents domed isodiametrical cells and sparse hydropotes(Fig. 2D).The stamen surface shows conical-papillate cells, with stomata mostly concentrated at the base (Figs. 2E,F).

Victoria cruziana *A.D.Orb.*

In *Victoria cruziana* the abaxial sepal surface presents a macromorphological differentiation between sepaloid green patches and petaloid white patches. The latter are distributed along the distal margin, in the areas that were covered in bud. In the sepaloid areas the epidermis

appears to be composed of tetragonal tabular pavement cells, with an extremely dense cover of hydropotes and sparse stomata (Fig.3A). In the proximal areas, uniseriate trichomes are present. The abaxial petaloid sepal areas and the adaxial sepal surface appear to have a similar micromorphology: they show tetragonal elongated, convex pavement cells, sparse stomata and hydropotes (Fig. 3B). Abaxial and adaxial surfaces both in exterior and interior petals share tetragonal, convex pavement cells, sparse hydropotes, stomata are absent on adaxial petal surfaces (Figs. 3C, D).

The staminodes show on abaxial and adaxial surfaces epidermis with tetragonal, convex, rugulate cells (Figs.3E, F), occasionally papillate cells are present; sparse hydropotes and stomata are present. External sterile and fertile stamens share epidermis with polygonal, papillate, ridged cells; stomata and hydropotes are present (Figs. 3G, H). Paracarpels show polygonal, papillate and ridged epidermis, stomata and hydropotes are absent. The tips of stamens are also devoid of hydropotes although stomata are present (Fig. 3I). Carpel tip show epidermis with polygonal cells, exposed anticlinal walls, devoid of both hydropotes and stomata.

Under TEM observations the connective of the stamens presents epidermal cells with thick cell wall (about 5µm). The total thickness at the papilla is about 12µm (Fig. 3J). Subepidermal cells show nuclei surrounded by amyloplasts (Fig. 3K). The thick cell wall is marked by small osmiophilic droplets and presents a thick cuticle (250-600 nm) bounded by osmiophilic material (Fig. 3L).

Nymphaea alba *L.*

Nymphaea alba shows little differentiation between organs of different series with regards to pavement cells. Abaxial sepal surfaces present tetragonal to polygonal tabular cells, with the anticlinal walls well evident; it shows anomocytic stomata and possesses sparse hydropotes

(Fig. 4A). The adaxial sepal surface shows tetragonal-elongated, slightly convex pavement cells; stomata and hydropotes are quite sparse (Fig. 4B). The petals don't show a great differentiation between the two surfaces: they both present tetragonal-elongated cells with lightly wavy anticlinal walls. Stomata appear to be absent, and hydropotes are quite sparse (Figs. 4C, D). The stamens present a light differentiation between connective and thecae epidermis with respect to cellular shape, with more elongated tetragonal cells in the latter; quite similar to petal epidermis, and more polygonal cells in the former (Figs. 4E).

Nymphaea gigantea *Hook.*

In *Nymphaea gigantea* (Fig. 4F) the stamen presents the same differentiation between thecae and connective and the same cell type as *N.caerulea*. There is a light differentiation in the shape of the connective pavement cells, more irregular and with curved anticlinal walls in *N.gigantea,* where the outline of the cells appears more marked.

Nymphaea caerulea *Savigny*

In *Nymphaea caerulea*, the abaxial sepal surface presents tetragonal to polygonal tabular cells. Stomata and hydropotes are also present (Fig.5A). The adaxial sepal surface presents tetragonal-elongated, slightly convex multipapillate cells that are also present in both the petal surfaces (Fig. 5B). Stomata are absent on abaxial and adaxial petal surfaces, while hydropotes are always present(Figs. 5C, D) Stamen surfaces are differentiated between the connective, including the apical protrusion, and the thecae, with more elongated cells in the latter; the cellular type is the same of the petals and the adaxial sepal surface. Stomata are absent, and hydropotes are present only on the abaxial connective and the apical extension (Fig. 5E).

Nymphaea lotus *L.*

The abaxial sepal surface in *Nymphaea lotus* presents polygonal tabular cells. Stomata are dense, and hydropotes sparse (Fig. 5F). On the adaxial surface of the sepal, the cells are faintly multipapillate, and stomata and hydropotes are equally sparse (Fig. 5G). The abaxial surface of the petal presents multipapillate tetragonal-elongated cells with wavy anticlinals. Hydropotes are sparse, and stomata rare (Fig. 5H). The adaxial surface of the petals presents very little differentiation from the abaxial. The pavement cells are tetragonal-elongate to polygonal (Fig. 5I). Stomata are concentrated on the abaxial side of the stamen, and on the base and apex of the structure, pavement cells are multipapillate. Hydropotes can be found on the abaxial side(Figs. 5J, K), where they are present on all the connective epidermis, and are particularly concentrated on the connective tip. The carpellary tip are devoid of stomata or hydropotes, and they present polygonal multipapillate cells(Fig. 5L).

Signal dissection and morphological congruence

The trees obtained from the MP analysis of the matrices are summarized in Table 2. The trimmed matrix from Borsch et al. (2008) and the fused matrix of Borsch et al. (2008) and Taylor et al. (2015) result in the most MP trees. These trees differ in the relationships between the *Nymphaea* species, *Victoria*, and *Euryale*, and result in an unresolved consensus. The addition of our three characters to both matrices results in a single MP tree. This tree shows a monophyletic *Nymphaea*, with *N.alba* as sister to *N.lotus* +(*N.gigantea* + *N.caerulea*).*Victoria* + *Euryale* is sister to *Nymphaea.* The trees from Matrix 2 and Matrix 4 show a generally lower level of homoplasy as shown by the higher Consistency Index and Retention Index (Table 2). The Delta scores for the different matrices show that the addiction of our characters results in an increase in signal tree-likeliness in both Matrix 2 and Matrix 4.

The bootstrap analysis shows that in the case of the Borsch et al. (2008) matrix, no node receives substantial support (Fig. S1A), and the addition of the characters from Taylor et al. (2015) does not improve this situation. The addition of our three characters to both matrices results in a modest increase in bootstrap support for the crown of *Nymphaea* (73 for the second matrix and 64 for the fourth), the tropical Nymphaeas (53 and 52), the sister relationship of *N.caerulea* and *N.gigantea*(53 and 53)as well as for the grouping of *Victoria* and *Euryale* (63 and 65) (Fig.S1B).

A similar pattern is seen in the Bayesian inference results. However, here the Borsch et al. (2008) matrix presents marginal support for a clade of *Euryale* and *Victoria* (0.67 posterior probability in the analysis with equal rates and 0.74 in the analysis with gamma-distributed rates) and for a clade of *N.caerulea* and *N.gigantea* (0.59 in the analysis with equal rates and 0.5 in the analysis with gamma-distributed rates). However, the signal for monophyly of *Nymphaea* is only marginally stronger than a signal for a clade of *Victoria cruziana* and *Nymphaea lotus* (Table3). The addition of our characters improves the support for the monophyly of tropical *Nymphaea*, the monophyly of the genus and the relationship between *Euryale* and *Victoria*. On the other hand, support for a link between *Victoria* and *Nymphaea lotus* disappears with the addition of our characters (Table 3).

Ancestral character state reconstruction

The tree potential topologies investigated show some differences in the reconstructed history of our three characters (Figs. 6), as well as different levels of homoplasy (Table S3). The presence of elongation of the pavement cells of the petaloid patches is reconstructed as synapomorphic of the genus *Nymphaea* in the topology were the genus is reconstructed as monophyletic (Fig. 6A); in the second topology, the elongated state has either evolved independently in *Nymphaea* subg *Nymphaea* or has been lost in the *Victoria-Euryale* clade

(Fig. 6C); in the third topology, the elongated state evolves before the split of subg *Nymphaea* and it's lost in the *Victoria-Euryale* clade (Fig. 6E). The epidermal differentiation between organ series follows the exact same pattern as the petal cell elongation.

The simple and multiple papillae are reconstructed as independent in the first topology, with the simple papillae being synapomorphic for the *Victoria-Euryale* clade and the multiple papillae being synapomorphic for the tropical Nymphaeas (Fig. 6B); in the second topology, the papillate states originate after the split of subg *Nymphaea*, but the ancestral state is ambiguous (Fig. 6D); in the third topology, the simple papillate state of *Victoria* and *Euryale* derives from a multipapillate state (Fig. 6F).

Discussion

Our data offer insights into the functional role of floral epidermis in the waterlilies and the influence of historical factors on its evolution. The morphology of the floral epidermis and degree of differentiation between the epidermis of studied organ series show a clear pattern between different groups of species. All species investigated present a differentiation between the outer sepaloid sepal surface and the other surfaces, as already noted by Warner et al. (2008, 2009). However, this sepaloid surface differs in some ways between *Euryale* and *Victoria*, where it is densely covered in trichomes and hydropotes, and the species of *Nymphaea,* where it is not. This difference in the distribution of the hydropotes between the sepaloid patches of the outer tepals in these two groups of species presents interesting parallelism with the distribution of these specialized cells in the leaves, given that *Victoria* and *Euryale* represent the only taxa in the Nymphaeales to have adaxial hydropotes on the leaf surfaces (Carpenter 2006). These two species also share an evident differentiation

between the cell types present in the epidermis of the different organ series, with sepals presenting tabular cells, petals presenting domed cells and stamens presenting conical-papillate cells. This latter cell type is quite rare among basal angiosperms, and has only been reported from *Austrobaileya* among ANA-grade taxa (Endress 1983). In *Victoria cruziana* the differentiation reaches its maximum, with extremely specialized conical-papillate cells with radially arranged folds being present on the epidermis of stamens and paracarpels.

The flowers of the species of the genus *Nymphaea* lack clear differentiation between the epidermal types in the different organ series. They also all share tetragonal-elongated cells in the petal epidermis. In the three tropical *Nymphaea* species, a multipapillate cell type is present in all organs, with the exclusion of the adaxial sepaloid surface of the outer tepals. This epidermal type has also been shown to be present in *Nymphaea amazonum* and *Nymphaea gardneriana* from subgenus *Hydrocallis* (Zini et al 2017).

Differentiation hierarchies in the waterlily flower

The study of organ identity determination in the waterlilies have mostly focused on the peculiarities of this group, like the presence of different patches in single organs (Warner et al. 2008, 2009). The Mosaic Model proposed for this group predicts that, in an early stage of the evolution, the features of sepalness and petalness were not fixed to particular organs, but controlled by an interaction of internal and environmental factors. This model went on to expand the so called "fading borders" model (Buzgo et al., 2004), that postulated a wide area of expression of the floral regulators of the (A)BCDE system (Theissen & Saedler, 2001; Theissen & Melzer, 2007), as reported by studies of gene expression in the waterlilies (Yoo, Soltis & Soltis, 2010; Yoo et al. 2010). Our observations confirm what Warner et al. (2008;2009) observed, and underline the necessity of an approach to gene expression study that would also take into consideration the spatial distribution of floral regulator expression in

the single organs. The strong differentiation of the organ series in *Victoria cruziana* and *Euryale ferox*, on the other hand, demonstrate a more sophisticated system of regulation, at least with regards to epidermal morphology. It seems that in this species the identity of the organs of the single series is more closely determined with respect to the other species of the water lilies, as reflected i.e. in the macromorphological "gap" between the outer staminodes and the stamens in *Victoria*. How this differentiation is possible, given the wide and relatively poorly differentiated expression of the MADS-box genes in the waterlilies (Geuten et al. 2006; Kim et al., 2004; Yoo, Soltis & Soltis, 2010; Yoo et al.2010) is an interesting and stimulating question. A potentially promising avenue for research involves the role of R2R3 MYB proteins in the differentiation of floral epidermis. These proteins have been involved in the development of conical cells in different models (Noda et al. 1994), including staminal epidermis of apetalous flowers (Di Stilio et al. 2009). Given the presence of multiple copies of the R2R3 MYB genes in the genome of *Nuphar advena* (Brockington et al. 2013), the differential expression of these genes in the epidermis of the more complex flowers of the Nymphaeaceae could be at the base of the differentiation of cell types.

Functional aspect of the epidermis in *Victoria cruziana*

The presence of the papillate-conical epidermis in the stamens and particularly in the paracarpels of *Victoria cruziana* and their peculiar ultrastructure seems to suggest a link with the sophisticated pollination biology of the genus (Valla & Cirino 1972: Prance & Arias 1975). A papillate-conical epidermis is a common feature in many angiosperm flowers (Kay et al. 1981; Whitney et al., 2011), and has even been used by various authors as a marker for petal identity (Irish 2009). The epidermal cells of *Victoria*, however, even if extremely similar in shape, are quite different with regard to their structure as compared to the conical-

papillate cells of other angiosperms. The papilla, that in normal conical-papillate cells represents a trichome-like extension of the cellular body (Kay et al. 1981; Effmert et al. 2005, 2006), appears to be formed by an unusual umbo-like thickening of the external periclinal cell wall (i.e. a solid dome-shaped thickening of the central part of the wall itself) as evident from TEM images. The ultrastructural observation of the thickening shows that, even if the cell wall is abnormally thick, it appears to be permeable to osmiophilic compounds, that could represent lipids, terpenoids or other volatile compounds. Moreover, the cuticle appears to be channelled, and osmiophilic substances are visible in the channels and deposited on the cuticle surface. The epidermal cells present a vacuole with osmiophilic deposits, and the underlying tissue presents plastids with plastoglobuli and starch granules, lipid droplets and large nuclei. This organization could indicate a potential secretory role for such an epidermal type. Even if staining with neutral red and olfactory analyses (Valla & Cirino 1972) originally suggested that stamens and paracarpels are not particularly involved in scent emission, at least during the first night of flowering, later investigations have shown that stamens present a similar staining behaviour to the inner tepals, and speculated about the functional role of the inner tepal epidermis (Zini et al. 2017). Given the scarcity of data correlating scent emission with ultrastructural or histological characters among basal angiosperms, we do not think that our data are sufficient to identify the connective of the stamens of *Victoria cruziana* as an osmophoric tissue (Baudino et al. 2007; Bell et al. 2009; Kolosova et al., 2001; Stern, Curry & Whitten 1986). A more detailed analysis of scent emission, which would involve an organ-specific analysis of scent emission trough time, is beyond the scope of the current investigation.

Testing homoplasies in floral morphology

Our results allow us to test our two hypotheses about anatomical specialization in the waterlilies. The species with shared pollination ecologies do not share particular anatomical characters. On the other hand, similar anatomical characters are shared by species with contrasting pollination ecologies, i.e. between *Victoria* and *Euryale,* or *Nymphaea lotus* and *Nymphaea caerulea*.

Our signal dissection analyses as well as our character reconstruction supports these conclusions, with our characters presenting tree-like phylogenetic signal. When combined with the extensive morphological matrix of Borsch et al. (2008), and the characters identified by Taylor et al. (2015) and Coiro & Barone Lumaga (2013), our characters increase the support for the grouping of *Victoria* and *Euryale,* the monophyly of *Nymphaea* and the grouping of the tropical Nymphaeas (subgenera *Lotos, Anecphya* and *Brachyceras*) using both MP and BI (Figure 5). Our characters also reduce the support for a night-blooming clade including *Victoria* and *Nymphaea lotus.* Moreover, the matrices that include our characters present lower Delta scores, indicating higher "tree-likeness" of the signal (Holland et al. 2002). These results all show that the anatomy of the floral organs in the Nymphaeaceae presents a rather strong phylogenetic signal, which complements and improves the signal present in larger matrices. Such signal manages to overcome the ecological signal probably present in some of the characters in the Borsch et al. (2008) matrix, such as pollination type and organ number. Moreover, the differences between matrices 2, 3, and 4 show that the anatomy of the floral organs is less labile compared to pollen characters (Taylor et al. 2015).

Our character reconstruction analyses indicate that some of our characters could indeed represent genuine synapomorphies for some of the clades of the Nymphaeaceae. The presence of multipapillate epidermis could be synapomorphic for a clade including all

tropical subgenera of *Nymphaea,*since it is present in all the subgenera investigated here as well as in subgenus *Hydrocallis* (Zini et al. 2017) and it is reconstructed to be ancestral to such clade in all three topologies. The conical-papillate epidermis of *Euryale* and *Victoria* represents another striking synapomorphy, given the level of homoplasy of this character across the angiosperms and its classical link with pollination (Whitney et al. 2011). The extreme specialization of the floral epidermis in *Victoria cruziana*, and the differences with the beetle-pollinated *Nymphaea lotus,* strengthens the hypothesis that such species adapted to cantarophyly (Krell et al., 2003) independently from each other, as suggested by some of the phylogenetic hypotheses on the group (Borsch et al 2008).

Though it should be noted that our taxon sampling is still relatively limited, and the characters identified here could still present a higher level of homoplasy over the whole diversity of the Nymphaeaceae. Nonetheless, they provide key insights in the anatomical basis of pollination strategies in Nymphaeaceae, and represent a promising avenue for further investigation in the structural and genetic basis of these traits, and in their evolution in the family.

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Figure captions

Figure 1. Relationships between the main clades of the angiosperms (A), main molecular hypotheses about the phylogeny of the Nymphaeaceae plus Cabombaceae (B-D), i.e. topology 1(B), topology 2 (C), and topology 3 (D), section of a flower of *Victoria cruziana* (E), and summary of the main hypotheses around the floral anatomy of the Nymphaeaceae (F). In E the carpels (Car), carpel tips (Ctips), paracarpels or inner staminodes (Pc), stamens (St), and petals (Pt) are indicated. In F, under hypothesis 1, closely related genera *Euryale* and *Victoria* share similar anatomy but contrastring pollination ecology. Under hypothesis 2, *Victoria* share more similarity with the night-blooming Nymphaeas.

Figure 2. *Nuphar lutea* (A): A, SEM image of stamen epidermis showing tetragonal elongated, lightly convex cells with wavy anticlinal walls. *Euryale ferox* (B-F): B, SEM image of sepal adaxial epidermis showing tetragonal, lightly convex pavement cells, sparse stomata (s) and hydropotes (h); C, SEM image of sepal adaxial surface showing hydropote (h); D, SEM image of petal epidermis showing tetragonal, convex pavement cells, and hydropotes (h); E, SEM image of the stamen abaxial surface showing conical-papillate cells; F, SEM image of stamen epidermis showing conical papillate cells (p), and stoma (s).Scale bars, 50µm (A); 20 µm (B,C); 30 µm (D); 100 µm (E); 10 µm (F).

Figure 3. *Victoria cruziana:* A, SEM image of sepal abaxial epidermis showing tetragonal to polygonal tabular cells with hydropotes (h) and stomata (s); B, SEM image of adaxial sepal surface showing tetragonal elongate, tabular to slightly convex cells, sparse hydropotes (h); C, SEM image of petal abaxial surface showing tetragonal, convex, rugulate cells, sparse hydropotes, stomata (s) are present; D, SEM image of petal adaxial epidermis showing tetragonal, convex pavement cells and sparse hydropotes (h); E, SEM image of staminode abaxial surface showing tetragonal, convex, rugulate, occasionally papillate cells, sparse hydropotes, stomata (s) are present; F, SEM detail of staminode adaxial surface showing rugulate surface of cells and papillate cells, stomata (s) present; G, SEM image of external sterile stamen adaxial surface showing polygonal, papillate ridged cells, hydropotes (h) and stomata (s) are present; H, SEM image of fertile stamen connective surface showing polygonal, papillate, ridged cells, hydropotes and stomata are absent; I, SEM image of fertile

stamen tipsurface showing polygonal, papillate, ridged cells and two anomocytic stomata (s); J, TEM image of fertile stamen epidermal cells showing cytoplasm bounded by osmiophilic material (*), thick cell wall with waved surface and a tangentially sectioned papilla; K, TEM image of fertile stamen subepidermal cells with plastids with starch granules (sg), lipid droplets (ld) and large nuclei (n); L, TEM image of fertile stamen cell wallpapilla showing channeled cuticle (cc) and extracellular secretion (es). Scale bars, 100µm (A-E, G, H); 10 µm (F, I, J); 5 µm (K); 250nm (L).

Figure 4. *Nymphaea alba* (A-E): A, SEM image of the sepal abaxial surface showing tetragonal to polygonal tabular cells, sparse hydropotes (h) and stomata (h); B, SEM image of the sepal adaxial surface showing tetragonal-elongated slightly convex cells, sparse hydropotes (h) and stomata; C, SEM image of the petal abaxial surface showing tetragonal-elongated slightly convex cells and sparse hydropotes, stomata are absent; D, SEM image of the petal adaxial surface showing tetragonal-elongated slightly convex cells and sparse hydropotes (h), stomata are absent; E, SEM image of the stamen connective surface showing tetragonal to polygonal cells, and hydropote (h), stomata are absent. *Nymphaea gigantea* (F): SEM image of the stamen connective surface showing polygonal multipapillate cells, stomata are absent.Scale bars, 100 µm (A-D); 50 µm (E); 10 µm (F).

Figure 5. *Nymphaea caerulea* (A-E): A, SEM image of the sepal abaxial surface showing tetragonal to polygonal tabular cells, hydropotes (h) and sparse stomata; B, SEM image of the sepal adaxial surface showing tetragonal-elongated multipapillate cells, dense hydropotes (h) and sparse stomata; C, SEM image of the petal abaxial surface showing tetragonal –elongated multipapillate cells, dense hydropotes (h) and sparse stomata; D, SEM image of the petal adaxial surface showing tetragonal-elongated multipapillated cells, dense hydropotes (h), stomata are absent; E, SEM image ofthe stamen tip surface showing polygonal multipapillate cells and hydropote (h), stomata are absent. *Nymphaea lotus* (F-L): F, SEM image of the sepal abaxial surface showing polygonal tabular cells, dense stomata (s) and sparse hydropotes (h); G, SEM image of the sepal adaxial surface showing tetragonal-multipapillate cells, sparse hydropotes (h) and stomata; H, SEM image of the petal abaxial surface showing tetragonal –elongated multipapillate cells with wavy anticlinal walls, sparse hydropotes (h) and rare stomata; I, SEM image of the petal adaxial surface showing tetragonal-elongated

multipapillated cells, sparse hydropotes (h), rare stomata; J, SEM image of the abaxial stamen surface showing polygonal multipapillate cells stomata and and hydropotes (h), pollen grains are visible (p); K, SEM image of the stamen adaxial surface showing polygonal multipapillate cells, hydropotes (h) and stomata (s); L, SEM image of the carpellary tip showing polygonal multipapillate cells. Scale bars, 200 µm (J); 100 µm (A-D, F, H,I,K); 50 µm (E); 30 µm (G); 10 µm (L).

Figure 6. Ancestral reconstruction of the Petaloid pavement cells character (Character 2) on the left and the Papillae character (Character 3) on the right using Parsimony. Character is reconstructed over a topology with monophyletic *Nymphaea* (A,B), a topology with *Victoria* and *Euryale* as sister to the tropical Nymphaeas (C,D), and a topology with *Victoria* and *Euryale* as sister to the night-blooming Nymphaeas (E,F).

Figure S1. Consensus networks of the bootstrap replicates from the Maximum Parsimony analysis of a) Matrix 1 (data from Borsch et al. 2008) and b) Matrix 2 (data from Borsch et al. 2008 plus our characters). Only splits present in more than 15% of the replicates are shown. Both the treelikeness of the signal and the support for the monophyly of *Nymphaea,* the *Victoria* plus *Euryale* clade and the tropical *Nymphaea* clade clearly improve from a to b.

Species	Organ	Surface	Epidermis	Hydropotes	Stomata
Nuphar lutea	*tepal	abaxial	tetragonal, tabular	present	present
	*tepal	adaxial	tetragonal	sparse	absent
	stamen	connective	tetragonal-elongated, slightly convex, wavy anticlinals	absent	absent
Euryale ferox	sepal	abaxial	tetragonal, tabular	dense	dense
		adaxial	tetragonal,slightly convex	sparse	absent
	petal		tetragonal, domed	sparse	absent
	stamen		conical-papillate cells	absent	sparse
Victoria cruziana	sepal	abaxial	Tetragonal, tabular	dense	dense
		abaxial petaloid	tetragonal-polygonal, tabular -slightly convex	sparse	sparse
		adaxial	Tetragonal, tabular	sparse	sparse
	petal	abaxial	tetragonal, domed	sparse	present
		adaxial	tetragonal, domed	sparse	absent
	interior petal	abaxial	tetragonal, domed	sparse	present
		adaxial	tetragonal, domed	sparse	absent
	staminode	abaxial	tetragonal, domed, occasionally papillate	sparse	present
		adaxial	tetragonal, domed , occasionally papillate	absent	present
	stamen, external	abaxial	polygonal, papillate, ridged	present	present
		adaxial	polygonal, papillate, ridged	present	present
	stamen, fertile	connective	polygonal, papillate, ridged	absent	absent
		tip	polygonal, papillate, ridged	absent	dense
	paracarpel		polygonal, papillate, ridged	absent	absent
Nymphaea alba	sepal	abaxial	tetragonal to polygonal, tabular	sparse	dense
		adaxial	tetragonal-elongated, slightly convex	sparse	sparse
	petal	abaxial	tetragonal-elongated, slightly convex	sparse	absent
		adaxial	tetragonal-elongated, slightly convex	sparse	absent
	stamen	connective	tetragonal to polygonal, slightly convex	sparse	absent

Nymphaea gigantea	stamen	connective	polygonal, multipapillate	sparse	absent
Nymphaea caerulea	sepal	abaxial	tetragonal to polygonal, tabular	present	sparse
		adaxial	tetragonal-elongated, multipapillate	dense	sparse
	petal	abaxial	tetragonal-elongated, multipapillate	dense	sparse
		adaxial	tetragonal-elongated, multipapillate	dense	absent
	stamen	connective	polygonal, multipapillate	absent	absent
		tip	tetragonal-elongated, multipapillate	present	absent
Nymphaea lotus	sepal	abaxial	polygonal tabular cell	sparse	dense
		adaxial	faintly multipapillate	sparse	sparse
	petal	abaxial	multipapillate tetragonal-elongated cells, wavy anticlinals	sparse	rare
		adaxial	multipapillate tetragonal-elongated cells, wavy anticlinals	sparse	rare
	stames	connettive	multipapillate	present	present
	carpellary tip		polygonal multipapillate	absent	absent

Table 1. Summary of the cells types and specialized cells in the different organs of the species examined. *Information for tepal epidermis in *N. lutea* from Warner et al, 2008.

	NUMBER OF TREES	LENGTH	CI	RI	Δ SCORE
MATRIX 1	9	81	0.7547	0.5938	0.3775
MATRIX 2	1	85	0.7719	0.6750	0.3196
MATRIX 3	15	82	0.7500	0.6000	0.3827
MATRIX 4	1	88	0.7705	0.6818	0.3339

Table 2. Description of the trees obtained in the Maximum parsimony analyses of the different matrices. Delta scores, calculated using SplitsTree, are also included.

	Matrix1 equal	*Matrix1 gamma*	*Matrix2 equal*	*Matrix2 gamma*	*Matrix3 equal*	*Matrix3 gamma*	*Matrix4 equal*	*Matrix4 gamma*
Brachyceras + *Anecphya*	0.59	0.50	0.51	0.51	0.48	0.45	0.12	0.48
Tropical *Nymphaeas*	0.39	0.31	0.52	0.49	0.30	0.27	0.47	0.45
Victoria + *Euryale*	0.67	0.74	0.76	0.85	0.70	0.81	0.81	0.88
Monophyletic *Nymphaea*	0.31	0.27	0.51	0.50	0.28	0.21	0.44	0.42
Night-blooming clade	0.20	0.12	0	0	0.10	0.9	0	0

Table 3. Support (posterior probability) for different hypotheses in the Bayesian inference analyses of the different matrices.

Appendix

Character 1: Epidermal differentiation between petals and stamens: 0) Absent or light; 1) Present.

Character 2: Epidermal cell elongation in the petals: 0) Elongated; 1) Isodiametrical.

Character 3: Papillae: 0) Absent; 1) Multiple per cell; 2) Single per cell.